TITLE OF THE INVENTION

ORGANIC NITROGEN-CONTAINING COMPOSITION AND FERTILIZER COMPRISING THE SAME

BACKGROUND OF THE INVENTION

The present invention relates to waste liquor of L-glutamic acid fermentation, which is usable as a raw material for a fertilizer, and a fertilizer comprising it.

L-Glutamic acid is produced mainly by fermentation utilizing so-called L-glutamic acid-producing coryneform bacteria belonging to the genus *Brevibacterium*, *Corynebacterium* or *Microbacterium* or mutant strains thereof (Amino Acid Fermentation, Gakkai Shuppan Center, pp.195-215, 1986). As methods for producing L-glutamic acid by fermentation by using other bacterial strains, there are known a method using a microorganism belonging to the genus *Bacillus*, *Streptomyces*, *Penicillium* or the like (U.S. Patent No. 3,220,929), a method using a microorganism belonging to the genus *Pseudomonas*, *Arthrobacter*, *Serratia*, *Candida* or the like (U.S. Patent No. 3,563,857), a method using a microorganism belonging to the genus *Bacillus*, *Pseudomonas*, *Serratia*, *Aerobacter aerogenes* (currently referred to as *Enterobacter aerogenes*) or the like (Japanese Patent Publication (Kokoku) No. 32-9393), a method using a mutant strain of *Escherichia coli* (Japanese Patent Application Laid-open (Kokai) No. 5-244970) and so forth. In addition, the inventors of

the present invention proposed a method for producing L-glutamic acid by using a microorganism belonging to the genus *Klebsiella, Erwinia* or *Pantoea* (Japanese Patent Application Laid-open No. 2000-106869).

Further, there have been disclosed various techniques for improving L-glutamic acid-producing ability by enhancing activities of L-glutamic acid biosynthetic enzymes through use of recombinant DNA techniques. For example, it was reported that introduction of a gene coding for citrate synthase derived from *Escherichia coli* or *Corynebacterium glutamicum* was effective for enhancement of L-glutamic acid-producing ability in *Corynebacterium* or *Brevibacterium* bacteria (Japanese Patent Publication (Kokoku) No. 7-121228). In addition, Japanese Patent Application Laid-open No. 61-268185 discloses a cell harboring recombinant DNA containing a glutamate dehydrogenase gene derived from *Corynebacterium* bacteria. Further, Japanese Patent Application Laid-open No. 63-214189 discloses a technique for increasing L-glutamic acid-producing ability by amplifying a glutamate dehydrogenase gene, an isocitrate dehydrogenase gene, an aconitate hydratase gene and a citrate synthase gene.

With respect to the method for producing L-glutamic acid as described above, mother liquor after recovery of L-glutamic acid has been used as a raw material of a fertilizer or the like (Japanese Patent Application Laid-open No. 50-129363, Japanese Patent

Publication No. 35-16965, Japanese Patent Application Laid-open No. 52-7872). Therefore, in the method for producing L-glutamic acid by fermentation, it is considered desirable not only that productivity of L-glutamic acid is improved but also that mother liquor which is more suitable for a raw material of a fertilizer is obtained.

There is known a method wherein fermentation is performed as L-amino acid accumulated in culture is crystallized (Japanese Patent Application Laid-open No. 62-288). In this method, the L-amino acid concentration in the culture is maintained below a certain level by precipitating the accumulated L-amino acid in the culture. Specifically, L-tryptophan, L-tyrosine or L-leucine is precipitated during fermentation by adjusting temperature and pH of the culture or adding a surfactant to a medium.

While a method of carrying out fermentation with precipitation of L-amino acid accompanied is known as described above, amino acids suitable for this method are those showing a relatively low water solubility, and no example of applying the method to highly water-soluble amino acids such as L-glutamic acid is known. In addition, the medium must have low pH to precipitate L-glutamic acid. However, L-glutamic acid-producing bacteria such as those mentioned above cannot grow under an acidic condition, and therefore L-glutamic acid fermentation is performed under neutral conditions (U.S. Patent Nos. 3,220,929 and 3,032,474; K.C. Chao &

J.W. Foster, J. Bacteriol., 77, pp.715-725 (1959)). Thus, production of L-glutamic acid by fermentation accompanied by precipitation is not known. Furthermore, it is known that growth of most acidophile bacteria is inhibited by organic acids such as acetic acid, lactic acid and succinic acid (Yasuro Oshima Ed., "Extreme Environment Microorganism Handbook", p.231, Science Forum; R.M. Borichewski, J. Bacteriol., 93, pp.597-599 (1967) etc.). Therefore, it is considered that many microorganisms are susceptible to L-glutamic acid, which is also an organic acid, under acidic conditions, and there has been no report that search of microorganisms showing L-glutamic acid-producing ability under acidic conditions was attempted.

SUMMARY OF THE INVENTION

Under the circumstances as described above, an object of the present invention is to provide fermentation mother liquor which is more suitable for use as a raw material of a fertilizer or the like, without reducing productivity of L-glutamic acid.

The inventors of the present invention found that fermentation mother liquor obtained by culturing a microorganism having L-glutamic acid-producing ability in a liquid medium of which pH is adjusted to a condition under which L-glutamic acid precipitates, to allow L-glutamic acid to be produced and accumulated in a medium with precipitation of L-glutamic acid accompanied, contains a large amount of organic

nitrogen and is a suitable material as a raw material of a fertilizer or the like. Thus, they accomplished the present invention.

The present invention provides the followings.

(1) An organic nitrogen-containing composition comprising fermentation mother liquor obtained by culturing a microorganism having L-glutamic acid-producing ability in a liquid medium of which pH is adjusted to a condition under which L-glutamic acid is allowed to be precipitated, to allow L-glutamic acid to be produced and accumulated with precipitation of L-glutamic acid accompanied, and then separating L-glutamic acid from the medium.

(2) The organic nitrogen-containing composition according to (1), wherein the microorganism belongs to the genus *Enterobacter*.

(3) The organic nitrogen-containing composition according to (2), wherein the microorganism is *Enterobacter agglomerans*.

(4) The organic nitrogen-containing composition according to any one of (1) to (3), wherein the microorganism can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source, at a specific pH, and has an ability to accumulate L-glutamic acid in an amount exceeding the saturation concentration of L-glutamic acid in the liquid medium at the pH.

(5) The organic nitrogen-containing composition

according to (4), wherein the specific pH is 5.0 or less.

(6) The organic nitrogen-containing composition according to (4) or (5), wherein a pH suitable for L-glutamic acid production by the microorganism is a pH at which L-glutamic acid precipitates in the medium, and L-glutamic acid is produced and accumulated with precipitation of the L-glutamic acid accompanied, during the culture in the medium at the pH.

(7) A fertilizer comprising the organic nitrogen-containing composition as defined in any one of (1) to (6).

According to the present invention, fermentation mother liquor suitable for a raw material of a fertilizer or the like can be efficiently produced by fermentation.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is a restriction enzyme map of a DNA fragment derived from *Enterobacter agglomerans* in pTWVEK101.

Fig. 2 shows comparison of an amino acid sequence deduced from a nucleotide sequence of an *sucA* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli* (upper: *Enterobacter agglomerans*, column: *Escherichia coli*, the same shall apply to the followings).

Fig. 3 shows comparison of an amino acid sequence deduced from a nucleotide sequence of an *sucB* gene

derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 4 shows comparison of an amino acid sequence deduced from a nucleotide sequence of an *sucC* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 5 shows comparison of an amino acid sequence deduced from a nucleotide sequence of an *sdhB* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 6 shows construction of a plasmid pMWCPG containing a *gltA* gene, a *ppc* gene and a *gdhA* gene.

Fig. 7 shows construction of a plasmid RSF-Tet containing a replication origin of a broad-host-range plasmid RSF1010 and a tetracycline resistance gene.

Fig. 8 shows construction of a plasmid RSFCPG containing a replication origin of a broad-host-range plasmid RSF1010, a tetracycline resistance gene, a *gltA* gene, a *ppc* gene and a *gdhA* gene.

Fig. 9 shows the construction of plasmid pSTVCB containing a *gltA* gene.

DETAILED DESCRIPTION OF THE INVENTION

Hereafter, the present invention will be explained in detail.

The organic nitrogen means nitrogen other than ammonia nitrogen among the total nitrogen. Generally, it is nitrogen contained in organic materials constituting amino acids, peptides, proteins, nucleic

acids and the like.

The organic nitrogen-containing composition of the present invention can be obtained as fermentation mother liquor obtained by culturing a microorganism having L-glutamic acid-producing ability in a liquid medium of which pH is adjusted to a condition under which L-glutamic acid is allowed to be precipitated, to allow L-glutamic acid to be produced and accumulated with precipitation of L-glutamic acid accompanied, and then separating L-glutamic acid from the medium.

Examples of the microorganism having L-glutamic acid-producing ability used in the present invention include microorganisms belonging to the genus *Enterobacter*. Preferred is *Enterobacter agglomerans*.

Further, the microorganism having L-glutamic acid-producing ability used in the present invention is preferably a microorganism that can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source, at a specific pH, and has an ability to accumulate L-glutamic acid in an amount exceeding the saturation concentration of L-glutamic acid in the liquid medium at the aforementioned pH (henceforth also referred to as "L-glutamic acid-accumulating microorganism"). The aforementioned specific pH is preferably a pH at which L-glutamic acid precipitates in the medium, and such a pH is usually 5.0 or less.

The "saturation concentration" means a concentration of L-glutamic acid dissolved in the

liquid medium when the liquid medium is saturated with L-glutamic acid.

When an L-glutamic acid-accumulating microorganism is used, the pH suitable for the production of L-glutamic acid is preferably a pH at which L-glutamic acid precipitates in the medium. By performing the culture at this pH, L-glutamic is produced and accumulated in the medium with its precipitation accompanied.

The L-glutamic acid-accumulating microorganism can be obtained as follows. A sample containing microorganisms is inoculated into a liquid medium containing L-glutamic acid at a saturation concentration and a carbon source, at a specific pH, and a strain that metabolizes the carbon source is selected. Although the specific pH is not particularly limited, it is usually about 5.0 or less, preferably about 4.5 or less, further preferably about 4.3 or less. The L-glutamic acid-accumulating microorganism is used for production of L-glutamic acid by fermentation with precipitation of the L-glutamic acid accompanied. If the pH is too high, it becomes difficult to allow the microorganism to produce L-glutamic acid in an amount sufficient for precipitation. Therefore, pH is preferably in the aforementioned range.

If pH of an aqueous solution containing L-glutamic acid is lowered, the solubility of L-glutamic acid significantly falls around pKa of γ-carboxyl group (4.25, 25°C). The solubility becomes the lowest at the

isoelectric point (pH 3.2) and L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated. While it depends on the medium composition, L-glutamic acid is dissolved in an amount of 10-20 g/L at pH 3.2, 30-40 g/L at pH 4.0 and 50-60 g/L at pH 4.7, at about 30°C. Usually pH does not need to be made 3.0 or lower, because the L-glutamic acid precipitating effect reaches its upper limit when pH goes below a certain value. However, pH may be 3.0 or less.

In addition, the expression that a microorganism "can metabolize a carbon source" means that it can proliferate or can consume a carbon source even though it cannot proliferate, that is, it indicates that it catabolizes a carbon source such as sugars or organic acids. Specifically, for example, if a microorganism proliferates when it is cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, particularly preferably pH 4.0, at an appropriate temperature, for example, 28°C, 37°C or 50°C, for 2 to 4 days, this microorganism can metabolize the carbon source in the medium. Further, for example, if a microorganism consume a carbon source even though the microorganism does not proliferate, when it is cultured in a synthetic liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0,

particularly preferably pH 4.0, at an appropriate temperature, for example, 28°C, 37°C or 50°C, for 2 to 4 days, the microorganism is a microorganism that can metabolize the carbon source in the medium.

The microorganism that can metabolize a carbon source include a microorganism that can grow in the aforementioned liquid medium.

Further, the expression that a microorganism "can grow" means that it can proliferate or can produce L-glutamic acid even though it cannot proliferate. Specifically, for example, if a microorganism proliferates when it is cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, particularly preferably pH 4.0, at an appropriate temperature, for example, 28°C, 37°C or 50°C, for 2 to 4 days, this microorganism can grow in the medium. Further, for example, if a microorganism increases an amount of L-glutamic acid in a synthetic liquid medium even though the microorganism does not proliferate, when the microorganism is cultured in the synthetic liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, particularly preferably pH 4.0, at an appropriate temperature, for example, 28°C, 37°C or 50°C, for 2 to 4 days, this microorganism is a microorganism that can grow in the medium.

The selection described above may be repeated two or more times under the same conditions or with changing pH or the concentration of L-glutamic acid. A selection for an early stage can be performed in a medium containing L-glutamic acid at a concentration lower than the saturation concentration, and thereafter a subsequent selection can be performed in a medium containing L-glutamic acid at a saturation concentration. Further, strains with favorable properties such as superior proliferation rate may be selected.

The L-glutamic acid-accumulating microorganism is a microorganism that has an ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration of L-glutamic acid in a liquid medium, in addition to the properties described above. The pH of the aforementioned liquid medium is preferably the same as or close to that of the medium used for screening a microorganism having the aforementioned properties. Usually, a microorganism becomes susceptible to L-glutamic acid at a high concentration as pH becomes lower. Therefore, it is preferred that pH is not low in view of resistance to L-glutamic acid, but low pH is preferred in view of production of L-glutamic acid with its precipitation accompanied. To satisfy these conditions, pH can be in the range of 3 to 5, preferably 4 to 5, more preferably 4 to 4.7, further preferably 4 to 4.5, particularly preferably 4.0 to 4.3.

As the L-glutamic acid-accumulating microorganism of or breeding materials therefor, there can be mentioned, for example, microorganisms belonging to the genus *Enterobacter*, *Klebsiella*, *Serratia*, *Pantoea*, *Erwinia*, *Escherichia*, *Corynebacterium*, *Alicyclobacillus*, *Bacillus*, *Saccharomyces* or the like. Among these, microorganisms belonging to the genus *Enterobacter* are preferred. Hereafter, the microorganism of the present invention will be explained mainly for microorganisms belonging to the genus *Enterobacter*. However, the microorganism is not limited to those belonging to the genus *Enterobacter*, and those belonging to other genera can be similarly used.

As a microorganism belonging to the *Enterobacter*, there can be specifically mentioned *Enterobacter agglomerans*, preferably the *Enterobacter agglomerans* AJ13355 strain. This strain was isolated from soil in Iwata-shi, Shizuoka, Japan as a strain that can proliferate in a medium containing L-glutamic acid and a carbon source at low pH.

The physiological properties of AJ13355 are shown below:

(1) Gram staining: negative

(2) Behavior against oxygen: facultative anaerobic

(3) Catalase: positive

(4) Oxidase: negative

(5) Nitrate-reducing ability: negative

(6) Voges-Proskauer test: positive

(7) Methyl Red test: negative

(8) Urease: negative

(9) Indole production: positive

(10) Motility: motile

(11) H_2S production in TSI medium: weakly active

(12) β-Galactosidase: positive

(13) Saccharide-assimilating property:

Arabinose: positive

Sucrose: positive

Lactose: positive

Xylose: positive

Sorbitol: positive

Inositol: positive

Trehalose: positive

Maltose: positive

Glucose: positive

Adonitol: negative

Raffinose: positive

Salicin: negative

Melibiose: positive

(14) Glycerose-assimilating property: positive

(15) Organic acid-assimilating property:

Citric acid: positive

Tartaric acid: negative

Gluconic acid: positive

Acetic acid: positive

Malonic acid: negative

(16) Arginine dehydratase: negative

(17) Ornithine decarboxylase: negative

(18) Lysine decarboxylase: negative

(19) Phenylalanine deaminase: negative

(20) Pigment formation: yellow

(21) Gelatin liquefaction ability: positive

(22) Growth pH: growth possible at pH 4, good growth at pH 4.5 to 7

(23) Growth temperature: good growth at 25°C, good growth at 30°C, good growth at 37°C, growth possible at 42°C, growth impossible at 45°C

Based on these bacteriological properties, AJ13355 was determined as *Enterobacter agglomerans*.

The *Enterobacter agglomerans* AJ13355 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (now, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) on February 19, 1998 and received an accession number of FERM P-16644. It was then transferred to an international deposition under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6614.

The L-glutamic acid-accumulating microorganism may be a microorganism originally having L-glutamic acid-producing ability or one having L-glutamic acid-producing ability imparted or enhanced by breeding through use of mutagenesis treatment, recombinant DNA techniques or the like.

The L-glutamic acid-producing ability can be imparted or enhanced by, for example, increasing activity of an enzyme that catalyzes a reaction for biosynthesis of L-glutamic acid. The L-glutamic acid-producing ability can also be enhanced by decreasing or eliminating activity of an enzyme that catalyzes a reaction which branches off from the biosynthetic pathway of L-glutamic acid and generates a compound other than L-glutamic acid.

As examples of the enzyme that catalyzes the reaction for biosynthesis of L-glutamic acid, there can be mentioned glutamate dehydrogenase (hereafter, also referred to as "GDH"), glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase (hereafter, also referred to as "CS"), phosphoenolpyruvate carboxylase (hereafter, also referred to as "PEPC"), pyruvate dehydrogenase, pyruvate kinase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and so forth. Among these enzymes, one, two or three of CS, PEPC and GDH are preferred. Further, it is preferred that the activities of all the three enzymes, CS, PEPC and GDH, are enhanced in the L-glutamic acid-accumulating microorganism. In particular, CS of *Brevibacterium lactofermentum* is preferred, because it does not suffer from inhibition by α-ketoglutaric acid, L-glutamic acid and NADH.

In order to enhance the activity of CS, PEPC or GDH, for example, a gene coding for CS, PEPC or GDH can be cloned on an appropriate plasmid and a host microorganism can be transformed with the obtained plasmid. The copy number of the gene coding for CS, PEPC or GDH (hereafter, abbreviated as "*gltA* gene", "*ppc* gene" and "*gdhA* gene", respectively) in the transformed strain cell increases, resulting in the increase of the activity of CS, PEPC or GDH.

The cloned *gltA*, *ppc* and *gdhA* genes are introduced into the aforementioned starting parent strain solely or in combination of arbitrary two or three kinds of them. When two or three kinds of the genes are introduced, two or three kinds of the genes may be cloned on one kind of plasmid and introduced into the host, or separately cloned on two or three kinds of plasmids that can coexist and introduced into the host.

Two or more kinds of genes coding for an enzyme of the same kind, but derived from different microorganisms, may be introduced into the same host.

The plasmids described above are not particularly limited so long as they are autonomously replicable in a cell of a microorganism belonging to, for example, the genus *Enterobacter* or the like. However, there can be mentioned, for example, pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218, pACYC177, pACYC184 and so forth. Besides these, vectors of phage DNA can also be used.

Transformation can be performed by, for example, the method of D.M. Morrison (Methods in Enzymology, 68, 326 (1979)), the method wherein permeability of recipient bacterium cells for DNA is increased by treating the cells with calcium chloride (Mandel M. and Higa A., J. Mol. Biol., 53, 159 (1970)), electroporation (Miller J.H., "A Short Course in Bacterial Genetics", Cold Spring Harbor Laboratory Press, U.S.A., 1992) or the like.

The activity of CS, PEPC or GDH can also be increased by allowing multiple copies of the *gltA* gene, the *ppc* gene or the *gdhA* gene to be present on chromosomal DNA of the aforementioned starting parent strain to be a host. In order to introduce multiple copies of the *gltA* gene, the *ppc* gene or the *gdhA* gene on chromosomal DNA of a microorganism belonging to the genus *Enterobacter* or the like, a sequence of which multiple copies are present on the chromosomal DNA, such as repetitive DNA and inverted repeats present at terminus of a transposable element, can be used. Alternatively, multiple copies of the genes can be introduced onto chromosomal DNA by utilizing transfer of a transposon containing the *gltA* gene, the *ppc* gene or the *gdhA* gene. As a result, the copy number of *gltA* gene, the *ppc* gene or the *gdhA* gene in a transformed strain cell is increased, and thus the activity of CS, PEPC or GDH is increased.

As organisms used as a source of the *gltA* gene, the *ppc* gene or the *gdhA* gene of which copy number is

to be increased, any organism can be used so long as it has activity of CS, PEPC or GDH. *Inter alia*, bacteria, which are prokaryotes, for example, those belonging to the genus *Enterobacter, Klebsiella, Erwinia, Pantoea, Serratia, Escherichia, Corynebacterium, Brevibacterium* or *Bacillus* are preferred. As specific examples, there can be mentioned *Escherichia coli, Brevibacterium lactofermentum* and so forth. The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained from chromosomal DNA of the microorganisms described above.

The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained by using a mutant strain which is deficient in the activity of CS, PEPC or GDH to isolate a DNA fragment that supplements its auxotrophy from chromosomal DNA of the aforementioned microorganism. Further, since the nucleotide sequences of these genes of *Escherichia* and *Corynebacterium* bacteria have already been elucidated (Biochemistry, 22, pp.5243-5249, (1983); J. Biochem., 95, pp.909-916, (1984); Gene, 27, pp.193-199, (1984); Microbiology, 140, pp.1817-1828, (1994); Mol. Gen. Genet., 218, pp.330-339, (1989); Molecular Microbiology, 6, pp.317-326, (1992)), they can also be obtained by PCR utilizing primers synthesized based on each nucleotide sequence and chromosomal DNA as a template.

The activity of CS, PEPC or GDH can also be increased by enhancing the expression of the *gltA* gene, the *ppc* gene or the *gdhA* gene, besides the aforementioned amplification of the genes. For example,

the expression can be enhanced by replacing a promoter for the *gltA* gene, the *ppc* gene or the *gdhA* gene with another stronger promoter. For example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, P_R promoter and P_L promoter of the lamda phage and so forth are known as strong promoters. The *gltA* gene, the *ppc* gene and the *gdhA* gene of which promoter is replaced are cloned on a plasmid and introduced into the host microorganism, or introduced onto the chromosomal DNA of the host microorganism by using repetitive DNA, inverted repeat, transposon or the like.

The activity of CS, PEPC or GDH can also be increased by replacing the promoter of the *gltA* gene, the *ppc* gene or the *gdhA* gene on the chromosome with another stronger promoter (see WO87/03006 and Japanese Patent Application Laid-open No. 61-268183), or inserting a strong promoter in the upstream of the coding sequence of each gene (see Gene, 29, pp.231-241 (1984)). Specifically, homologous recombination can be performed between the *gltA* gene, the *ppc* gene or the *gdhA* gene of which promoter is replaced with a stronger one or DNA containing a part thereof and the corresponding gene on the chromosome.

Examples of the enzyme that catalyzes the reaction which branches off from the biosynthetic pathway of the L-glutamic acid and generates a compound other than L-glutamic acid include α-ketoglutarate dehydrogenase (hereafter, also referred to as "αKGDH"), isocitrate lyase, phosphate acetyltransferase, acetate

kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and so forth. Among these enzymes, αKGDH is preferred.

In order to decrease or eliminate the activities of the aforementioned enzymes in a microorganism belonging to the genus *Enterobacter* or the like, mutations for decreasing or eliminating the intracellular activity of the enzymes can be introduced into genes of the aforementioned enzymes by a usual mutagenesis treatment method or a genetic engineering method.

Examples of the mutagenesis treatment method include, for example, methods utilizing irradiation with X-ray or ultraviolet ray, methods utilizing treatment with a mutagenesis agent such as N-methyl-N'-nitro-N-nitrosoguanidine, and so forth. The site of a gene where the mutation is introduced may be in a coding region coding for an enzyme protein or a region for regulating expression such as a promoter.

Examples of the genetic engineering methods include, for example, methods utilizing gene recombination, transduction, cell fusion and so forth. For example, a drug resistance gene is inserted into a cloned target gene to prepare a gene that has lost its function (defective gene). Subsequently, this defective gene is introduced into a cell of a host microorganism, and the target gene on the chromosome is

replaced with the aforementioned defective gene by utilizing homologous recombination (gene disruption).

Decrease or deficiency of intracellular activity of the target enzyme and the degree of decrease of the activity can be confirmed by measuring the enzyme activity of a cell extract or a purified fraction thereof obtained from a candidate strain and comparing it with that of a wild strain. For example, the αKGDH activity can be measured by the method of Reed et al. (Reed L.J. and Mukherjee B.B., Methods in Enzymology, 13, pp.55-61 (1969)).

Depending on the target enzyme, a target mutant strain can be selected based on a phenotype of the mutant strain. For example, a mutant strain wherein the αKGDH activity is eliminated or decreased cannot proliferate or shows a markedly reduced proliferation rate in a minimal medium containing glucose or a minimal medium containing acetic acid or L-glutamic acid as an exclusive carbon source under an aerobic culture condition. However, normal proliferation is enabled even under the same condition by adding succinic acid or lysine, methionine and diaminopimelic acid to a minimal medium containing glucose. By utilizing these phenomena as indicators, a mutant strain with decreased αKGDH activity or deficient in the activity can be selected.

A method for preparing an αKGDH gene-deficient strain of *Brevibacterium lactofermentum* by utilizing homologous recombination is described in detail in

WO95/34672. Similar methods can be applied to other microorganisms.

Further, techniques such as cloning of genes and digestion and ligation of DNA, transformation and so forth are described in detail in Molecular Cloning, 2nd Edition, Cold Spring Harbor Press (1989) and so forth.

As a specific example of a mutant strain deficient in αKGDH activity or with decreased αKGDH activity obtained as described above, there can be mentioned *Enterobacter agglomerans* AJ13356. *Enterobacter agglomerans* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (now, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) on February 19, 1998 and received an accession number of FERM P-16645. It was then transferred to an international deposition under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6615. The *Enterobacter agglomerans* AJ13356 is deficient in αKGDH activity as a result of disruption of the αKGDH-E1 subunit gene (*sucA*).

When *Enterobacter agglomerans*, which is an example of the microorganism used in the present invention, is cultured in a medium containing a saccharide, mucus is extracellularly secreted, occasionally resulting in low operation efficiency. Therefore, when *Enterobacter agglomerans* having such a

property of secreting mucus is used, it is preferable to use a mutant strain that secretes less mucus compared with a wild strain. Examples of mutagenesis treatment include, for example, methods utilizing irradiation with X-ray or ultraviolet ray, method utilizing treatment with a mutagenesis agent such as N-methyl-N'-nitro-N-nitrosoguanidine, and so forth. A mutant strain with decreased secretion of mucus can be selected by inoculating mutagenized bacterial cells in a medium containing a saccharide, for example, LB medium plate containing 5 g/L of glucose, culturing them with tilting the plate about 45 degrees and selecting a colony that does not show flowing down of mucus.

In the present invention, impartation or enhancement of L-glutamic acid-producing ability and impartation of other favorable properties such as mutation for less mucus secretion described above can be carried out in an arbitrary order.

By culturing the microorganism having L-glutamic acid-producing ability in a liquid medium that is adjusted to pH condition that allows precipitation of L-glutamic acid, L-glutamic acid can be produced and accumulated with its precipitation in the medium accompanied.

Preferably, by culturing the L-glutamic acid-accumulating microorganism in a liquid medium that is adjusted to pH condition that allows precipitation of L-glutamic acid, L-glutamic acid can be produced and

accumulated with its precipitation in the medium accompanied. Furthermore, it is possible that the culture is started at a neutral pH, and pH becomes the condition that allows precipitation of L-glutamic acid when the culture is completed.

The "condition that allows precipitation of L-glutamic acid" referred to herein means a condition that allows precipitation of L-glutamic acid when the above-mentioned microorganism produces and accumulates L-glutamic acid. For example, it is usually 3 to 5 when the microorganism is an *Enterobacter* bacterium.

The microorganism may be cultured at pH suitable for growth thereof at the beginning and then cultured under the condition which allows precipitation of L-glutamic acid. For example, when the medium contains a sugar source which the microorganism can not assimilate under the condition which allows precipitation of L-glutamic acid, or an organic acid which inhibits the growth of the microorganism under the condition which allows precipitation of L-glutamic acid, the microorganism may be cultured under a condition under which the microorganism can assimilate the sugar source or growth of the microorganism is not inhibited by the organic acid to allow the microorganism to consume the sugar source or the organic acid, and then cultured under the condition allows precipitation of L-glutamic acid.

In a preferred embodiment of the present invention, during the culture, an operation causing

existence of L-glutamic acid crystals in the medium is performed when a concentration of L-glutamic acid in the medium is lower than the concentration at which spontaneous crystallization occurs.

The terms "spontaneous crystallization" used herein means that due to accumulation of L-glutamic acid by the microorganism having L-glutamic acid-producing ability, a concentration of L-glutamic acid in the medium exceeds a saturation concentration and L-glutamic acid spontaneously precipitates in the medium.

The operation causing existence of L-glutamic acid crystals in the medium means an operation by which existence of the crystals is artificially caused. Examples of the operation include addition of the crystals to the medium, and forced precipitation by lowering, during culture, pH of a medium in which a certain amount of L-glutamic acid has been dissolved at the beginning of the culture.

The amount of crystals to be existed in the medium is usually 0.01 to 10 g/L. The time at which the crystals are to be existed is preferably when the accumulated amount of L-glutamic acid in the medium increases to around the saturation concentration (for example, 25 g/L or more at pH 4.5). The amount of crystals existing in the medium and the concentration of L-glutamic acid can be determined by methods known to one skilled in the art. The existing amount of the crystals of L-glutamic acid may be determined by allowing the medium to stand and isolating the crystals

from the medium by decantation. The concentration of L-glutamic acid in the medium means a concentration of dissolved L-glutamic acid. When crystals precipitate in the medium, the concentration is a determined concentration of a clarified solution obtained by separating solids by centrifugation (or filtration).

The operation causing existence of L-glutamic acid crystals is preferably addition of L-glutamic acid crystals.

As for the L-glutamic acid crystals, there are α-form and β-form of crystals (H. Takahashi, T. Takenishi, N. Nagashima, Bull. Chem. Soc. Japan, 35, 923 (1962); J. D. Bernal, Z. Krist., 78, 363 (1931); S. Hirokawa, Acta Cryst., 8, 637 (1955)). When it is intended to obtain α-form of crystals, the crystals to be added are preferably of α-form.

A preferred amount of crystals varies depending on conditions such as crystal form of crystals. If the crystals are of α-form, it is usually 0.2 g/L or more. If it is more than this concentration, crystals of α-form may be obtained with good reproducibility. Because of their shape, the crystals of α-form may be handled more easily compared with crystals of β-form.

As the media used for culture, a usual nutrient medium containing a carbon source, a nitrogen source, mineral salts and organic trace nutrients such as amino acids and vitamins as required can be used so long as pH is adjusted so as to satisfy the predetermined condition. Either a synthetic medium or a natural

medium can be used. The carbon source and the nitrogen source used in the medium can be any ones so long as they can be used by the strain to be cultured.

As the carbon source, saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses are used. In addition, organic acids such as acetic acid and citric acid may be used each alone or in combination with another carbon source.

As the nitrogen source, ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate and ammonium acetate, nitrates and so forth are used.

As the organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, those containing these substances such as peptone, casamino acid, yeast extract and soybean protein decomposition products are used. When an auxotrophic mutant strain that requires an amino acid and so forth for metabolization or growth is used, the required nutrient must be supplemented.

As mineral salts, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and so forth are used.

The culture is usually performed with aeration under the condition of a culture temperature at 20 to 42°C, and pH at 3 to 5, preferably 4 to 5, more preferably 4 to 4.7, particularly preferably 4 to 4.5. A considerable amount of L-glutamic acid is usually accumulated after culture of from about 10 hours to

about 4 days. A portion of the accumulated L-glutamic acid which exceeds the saturation concentration precipitates in the medium.

After completion of the culture, L-glutamic acid precipitated in the culture can be collected by centrifugation, filtration or the like. L-Glutamic acid dissolved in the medium can be also collected by known methods. For example, the L-glutamic acid can be isolated by concentrating the culture broth to crystallize it or isolated by ion exchange chromatography or the like. It is also possible to crystallize L-glutamic acid dissolved in the medium and then collect the L-glutamic acid precipitated in the culture broth together with the crystallized L-glutamic acid.

The fermentation mother liquor obtained by separating L-glutamic acid may be used as an organic nitrogen-containing composition.

In the organic nitrogen-containing composition of the present invention, a content of organic nitrogen with respect to total nitrogen is high. Percentage by mass of organic nitrogen with respect to total nitrogen is preferably 35% or more.

In addition, according to the present invention, because L-glutamic acid is produced at a low pH, a used amount of ammonia for controlling pH of the medium becomes small, and an amount of an acid used for crystallization of L-glutamic acid accordingly becomes small, resulting in reduction of an amount of anion in

the fermentation mother liquor. Generally, the acid used therefor is an inorganic acid such as hydrochloric acid and sulfuric acid. For example, if sulfuric acid is used for crystallization of L-glutamic acid, an amount of sulfate group in the fermentation mother liquor becomes small. Percentage by mass of sulfate group with respect to total nitrogen is preferably 500% or less. As for a fertilizer, it is preferable that the amount of anion such as sulfate group is small. Therefore, the organic nitrogen-containing composition of the present invention is suitable for a raw material of a fertilizer.

The organic nitrogen-containing composition of the present invention may be liquid as the fermentation mother liquor, or may be made to be neutral dry granules by neutralizing and drying (see Japanese Patent Application Laid-open No. 52-7872).

Production of the fertilizer comprising the organic nitrogen-containing composition of the present invention can be performed similarly to production of a fertilizer using a conventional fermentation mother liquor as a raw material. During the production, another fertilizer ingredient may be added. Because the fertilizer of the present invention uses the organic nitrogen-containing composition of the present invention as a raw material, the fertilizer can have a high content of organic nitrogen (in particular, organic nitrogen other than nitrogen of L-glutamic acid) and a low content of anion such as sulfate group.

EXAMPLES

Hereafter, the present invention will be more specifically explained with reference to the following examples. In the examples, amino acids are L-amino acids unless otherwise indicated.

Reference Example 1

<1> Screening of microorganism having L-glutamic acid resistance in acidic environment

Screening of a microorganism having L-glutamic acid resistance in acidic environment was performed as follows. One (1) g each of about 500 samples obtained from nature including soil, fruits, plant bodies, river water and so forth was suspended in 5 mL of sterilized water, and 200 μL thereof was coated on 20 mL of solid medium adjusted to pH 4.0 with HCl. The composition of the medium was as follows: 3 g/L of glucose, 1 g/L of ammonium sulfate, 0.2 g/L of magnesium sulfate heptahydrate, 0.5 g/L of potassium dihydrogenphosphate, 0.2 g/L of sodium chloride, 0.1 g/L of calcium chloride dihydrate, 0.01 g/L of ferrous sulfate heptahydrate, 0.01 g/L of manganese sulfate tetrahydrate, 0.72 mg/L of zinc sulfate dihydrate, 0.64 mg/L of copper sulfate pentahydrate, 0.72 mg/L of cobalt chloride hexahydrate, 0.4 mg/L of boric acid, 1.2 mg/L of sodium molybdate dihydrate, 50 μg/L of biotin, 50 μg/L of calcium pantothenate, 50 μg/L of folic acid, 50 μg/L of inositol, 50 μg/L of niacin, 50 μg/L of p-aminobenzoic

acid, 50 μg/L of pyridoxine hydrochloride, 50 μg/L of riboflavin, 50 μg/L of thiamin hydrochloride, 50 mg/L of cycloheximide and 20 g/L of agar.

The media plated with the above samples were incubated at 28°C, 37°C or 50°C for 2 to 4 days and 378 strains forming colonies were obtained.

Subsequently, each of the strains obtained as described above was inoculated in a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of liquid medium (adjusted to pH 4.0 with HCl) containing a saturation concentration of L-glutamic acid and cultured at 28°C, 37°C or 50°C for 24 hours to 3 days with shaking. Then, the grown strains were selected. The composition of the aforementioned medium was follows: 40 g/L of glucose, 20 g/L of ammonium sulfate, 0.5 g/L of magnesium sulfate heptahydrate, 2 g/L of potassium dihydrogenphosphate, 0.5 g/L of sodium chloride, 0.25 g/L of calcium chloride dihydrate, 0.02 g/L of ferrous sulfate heptahydrate, 0.02 g/L of manganese sulfate tetrahydrate, 0.72 mg/L of zinc sulfate dihydrate, 0.64 mg/L of copper sulfate pentahydrate, 0.72 mg/L of cobalt chloride hexahydrate, 0.4 mg/L of boric acid, 1.2 mg/L of sodium molybdate dihydrate and 2 g/L of yeast extract.

Thus, 78 strains of microorganisms showing L-glutamic acid resistance in an acidic environment were successfully obtained.

<2> Selection of strains showing superior growth rate

from microorganisms having L-glutamic acid resistance in acidic environment

The various microorganisms having L-glutamic acid resistance in an acidic environment obtained as described above are each inoculated into a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of medium (adjusted to pH 4.0 with HCl) obtained by adding 20 g/L of glutamic acid and 2 g/L of glucose to M9 medium (Sambrook, J., Fritsh, E.F. and Maniatis, T., "Molecular Cloning", Cold Spring Harbor Laboratory Press, U.S.A., 1989), and the turbidity of the medium was measured in the time course to select strains showing a favorable growth rate. As a result, as a strain showing favorable growth, the AJ13355 strain was obtained from soil in Iwata-shi, Shizuoka, Japan. This strain was determined as *Enterobacter agglomerans* based on its bacteriological properties described above.

<3> Acquisition of strain with less mucus secretion from *Enterobacter agglomerans* AJ13355 strain

Since the *Enterobacter agglomerans* AJ13355 strain extracellularly secretes mucus when cultured in a medium containing a saccharide, operation efficiency is not favorable. Therefore, a strain with less mucus secretion was obtained by the ultraviolet irradiation method (Miller, J.H. et al., "A Short Course in Bacterial Genetics; Laboratory Manual", p.150, 1992, Cold Spring Harbor Laboratory Press, U.S.A.).

The *Enterobacter agglomerans* AJ13355 strain was

irradiated with ultraviolet ray for 2 minutes at a position 60 cm away from a 60-W ultraviolet lamp and cultured in LB medium overnight to fix mutation. The mutagenized strain was diluted and inoculated in LB medium containing 5 g/L of glucose and 20 g/L of agar so that about 100 colonies per plate would emerge and cultured at 30°C overnight with tilting the plate about 45 degrees, and then 20 colonies showing not flowing down of mucus were selected.

As a strain satisfying conditions that no revertant emerged even after 5 times of subculture in LB medium containing 5 g/L of glucose and 20 g/L of agar, and that there should be observed growth equivalent to the parent strain in LB medium, LB medium containing 5 g/L of glucose and M9 medium (Sambrook, J. et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Press, U.S.A., 1989) supplemented with 20 g/L of L-glutamic acid and 2 g/L of glucose and adjusted to pH 4.5 with HCl, SC17 strain was selected from the strains selected above.

<4> Construction of glutamic acid-producing bacterium from *Enterobacter agglomerans* SC17 strain

(1) Preparation of αKGDH deficient strain from *Enterobacter agglomerans* SC17 strain

A strain that was deficient in αKGDH and had enhanced L-glutamic acid biosynthetic system was prepared from the *Enterobacter agglomerans* SC17 strain.

(i) Cloning of αKGDH gene (hereafter, referred to as

"sucAB") of *Enterobacter agglomerans* AJ13355 strain

The *sucAB* gene of the *Enterobacter agglomerans* AJ13355 strain was cloned by selecting a DNA fragment complementing the acetic acid-unassimilating property of the αKGDH-E1 subunit gene (hereafter, referred to as *"sucA"*)-deficient strain of *Escherichia coli* from chromosomal DNA of the *Enterobacter agglomerans* AJ13355 strain.

The chromosomal DNA of the *Enterobacter agglomerans* AJ13355 strain was isolated by a method usually employed for extracting chromosomal DNA from *Escherichia coli* (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, pp.97-98, Baifukan, 1992). The pTWV228 (resistant to ampicillin) used as a vector was a commercial product of Takara Shuzo Co., Ltd.

The chromosomal DNA of the AJ13355 strain digested with *Eco*T221 and pTWV228 digested with *Pst*I were ligated by using T4 ligase and used to transform the *sucA*-deficient *Escherichia coli* JRG465 strain (Herbert, J. et al., Mol. Gen. Genetics, 105, 182 (1969)). A strain growing in an acetate minimal medium was selected from the transformant strains obtained above, and a plasmid was extracted from it and designated as pTWVEK101. The *Escherichia coli* JRG465 strain harboring pTWVEK101 recovered auxotrophy for succinic acid or L-lysine and L-methionine besides the trait of acetic acid-unassimilating property. This suggests that pTWVEK101 contained the *sucA* gene of

Enterobacter agglomerans.

Fig. 1 shows a restriction enzyme map of a DNA fragment derived from *Enterobacter agglomerans* in pTWVEK101. In the nucleotide sequence of the hatched portion in Fig. 1, nucleotide sequences considered to be two full length ORFs and two nucleotide sequences considered to be partial sequences of ORFs were found. As a result of homology search for these, it was revealed that the portions of which nucleotide sequences were determined contained a 3′ end partial sequence of the succinate dehydrogenase iron-sulfur protein gene (*sdhB*), full length *sucA* and αKGDH-E2 subunit gene (*sucB* gene), and a 5′ end partial sequence of the succinyl CoA synthetase β subunit gene (*sucC* gene). The results of comparison of the amino acid sequences deduced from these nucleotide sequences with those derived from *Escherichia coli* (Eur. J. Biochem., 141, pp.351-359 (1984); Eur. J. Biochem., 141, pp.361-374 (1984); Biochemistry, 24, pp.6245-6252 (1985)) are shown in Figs. 2 to 5. Thus, the amino acid sequences showed very high homology to each other. In addition, it was found that a cluster of *sdhB-sucA-sucB-sucC* was constituted on the chromosome of *Enterobacter agglomerans* as in *Escherichia coli* (Eur. J. Biochem., 141, pp.351-359 (1984); Eur. J. Biochem., 141, pp.361-374 (1984); Biochemistry, 24, pp.6245-6252 (1985)).

(ii) Acquisition of αKGDH-deficient strain derived from *Enterobacter agglomerans* SC17 strain

The homologous recombination was performed by using the *sucAB* gene of *Enterobacter agglomerans* obtained as described above to obtain an αKGDH-deficient strain of *Enterobacter agglomerans*.

After pTWVEK101 was digested with *Sph*I to excise a fragment containing *sucA*, the fragment was blunt-ended with Klenow fragment (Takara Shuzo Co., Ltd.) and ligated with pBR322 digested with *Eco*RI and blunt-ended with Klenow fragment, by using T4 DNA ligase (Takara Shuzo Co., Ltd.). The obtained plasmid was digested at the restriction enzyme *Bgl*II recognition site positioned approximately at the center of *sucA* by using the enzyme, blunt-ended with Klenow fragment, and then ligated again by using T4 DNA ligase. It was considered that the *sucA* gene became unfunctional because a frameshift mutation was introduced into *sucA* of the plasmid newly constructed through the above procedure.

The plasmid constructed as described above was digested with a restriction enzyme *Apa*LI, and subjected to agarose gel electrophoresis to recover a DNA fragment containing *sucA* into which the frameshift mutation was introduced and a tetracycline resistance gene derived from pBR322. The recovered DNA fragment was ligated again by using T4 DNA ligase to construct a plasmid for disrupting the αKGDH gene.

The plasmid for disrupting the αKGDH gene obtained as described above was used to transform the *Enterobacter agglomerans* SC17 strain by electroporation

(Miller, J.H., "A Short Course in Bacterial Genetics; Handbook", p.279, Cold Spring Harbor Laboratory Press, U.S.A., 1992), and a strain wherein *sucA* on the chromosome was replaced with a mutant type one of the plasmid by homologous recombination was obtained by using the tetracycline resistance as a marker. The obtained strain was designated as SC17sucA strain.

In order to confirm that the SC17sucA strain was deficient in the αKGDH activity, the enzyme activity was measured by the method of Reed et al. (Reed, L.J. and Mukherjee, B.B., Methods in Enzymology, 13, pp.55-61, (1969)) by using cells of the strain cultured in LB medium to the logarithmic growth phase. As a result, αKGDH activity of 0.073 (ΔABS/min/mg protein) was detected from the SC17 strain, whereas no αKGDH activity was detected from the SC17sucA strain, and thus it was confirmed that the *sucA* was eliminated as intended.

(2) Enhancement of L-glutamic acid biosynthesis system of *Enterobacter agglomerans* SC17sucA strain

Subsequently, the citrate synthase gene, phosphoenolpyruvate carboxylase gene and glutamate dehydrogenase gene derived from *Escherichia coli* were introduced into the SC17sucA strain.

(i) Preparation of plasmid having *gltA* gene, *ppc* gene and *gdhA* gene derived from *Escherichia coli*

The procedures of preparing a plasmid having the

gltA gene, the *ppc* gene and the *gdhA* gene will be explained by referring to Figs. 6 and 7.

A plasmid having the *gdhA* gene derived from *Escherichia coli,* pBRGDH (Japanese Patent Application Laid-open No. 7-203980), was digested with *Hin*dIII and *Sph*I, the both ends were blunt-ended by the T4 DNA polymerase treatment, and then the DNA fragment having the *gdhA* gene was purified and recovered. Separately, a plasmid having the *gltA* gene and *ppc* gene derived from *Escherichia coli,* pMWCP (WO97/08294), was digested with *Xba*I, and then the both ends were blunt-ended by using T4 DNA polymerase. This was mixed with the above purified DNA fragment having the *gdhA* gene and ligated by using T4 ligase to obtain a plasmid pMWCPG, which corresponded to pMWCP further containing the *gdhA* gene (Fig. 6).

At the same time, the plasmid pVIC40 (Japanese Patent Application Laid-open No. 8-047397) having the replication origin of the broad-host-range plasmid RSF1010 was digested with *Not*I, treated with T4 DNA polymerase and digested with *Pst*I. pBR322 was digested with *Eco*T14I, treated with T4 DNA polymerase and digested with *Pst*I. The both products were mixed and ligated by using T4 ligase to obtain a plasmid RSF-Tet having the replication origin of RSF1010 and the tetracycline resistance gene (Fig. 7).

Subsequently, pMWCPG was digested with *Eco*RI and *Pst*I, and a DNA fragment having the *gltA* gene, the *ppc* gene and the *gdhA* gene was purified and recovered.

RSF-Tet was similarly digested with *Eco*RI and *Pst*I, and a DNA fragment having the replication origin of RSF1010 was purified and recovered. The both products were mixed and ligated by using T4 ligase to obtain a plasmid RSFCPG, which corresponded to RSF-Tet containing the *gltA* gene, the *ppc* gene and the *gdhA* gene (Fig. 8). It was confirmed that the obtained plasmid RSFCPG expressed the *gltA* gene, the *ppc* gene and the *gdhA* gene based on the supplementation of the auxotrophy of the *gltA* gene-, *ppc* gene- or *gdhA* gene-deficient strain derived from *Escherichia coli* and measurement of each enzyme activity.

(ii) Preparation of plasmid having *gltA* gene derived from *Brevibacterium lactofermentum*

A plasmid having the *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using the primer DNAs which were prepared based on the nucleotide sequence of the *Corynebacterium glutamicum gltA* gene (Microbiology, 140, pp.1817-1828 (1994)), and chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template to obtain a *gltA* gene fragment of about 3 kb. This fragment was inserted into a plasmid pHSG399 (purchased from Takara Shuzo Co., Ltd.) digested with *Sma*I to obtain a plasmid pHSGCB (Fig. 9). Subsequently, pHSGCB was digested with *Hin*dIII, and the excised *gltA* gene fragment of about 3 kb was inserted into a plasmid pSTV29 (purchased from Takara Shuzo Co., Ltd.) digested

with *Hind*III to obtain a plasmid pSTVCB (Fig. 9). It was confirmed that the obtained plasmid pSTVCB expressed the *gltA* gene by measuring the enzyme activity in the *Enterobacter agglomerans* AJ13355 strain.

(iii) Introduction of RSFCPG and pSTVCB into SC17sucA strain

The *Enterobacter agglomerans* SC17sucA strain was transformed with RSFCPG by electroporation to obtain a transformant SC17sucA/RSFCPG strain showing tetracycline resistance. Further, the SC17sucA/RSFCPG strain was transformed with pSTVCB by electroporation to obtain a transformant SC17sucA/RSFCPG+pSTVCB strain showing chloramphenicol resistance.

<5> Acquisition of strain with improved resistance to L-glutamic acid in low pH environment

A strain with improved resistance to L-glutamic acid at a high concentration in a low pH environment (hereafter, also referred to as "strain with high-concentration Glu-resistance at low pH") was isolated from the *Enterobacter agglomerans* SC17sucA/RSFCPG+pSTVCB strain.

The SC17sucA/RSFCPG+pSTVCB strain was cultured overnight at 30°C in LBG medium (10 g/L of trypton, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose), and the cells washed with saline was appropriately diluted and plated on an M9-E medium (4 g/L of glucose, 17 g/L of $Na_2HPO_4 \cdot 12H_2O$, 3 g/L of KH_2PO_4, 0.5 g/L of NaCl,

1 g/L of NH_4Cl, 10 mM of $MgSO_4$, 10 μM of $CaCl_2$, 50 mg/L of L-lysine, 50 mg/L of L-methionine, 50 mg/L of DL-diaminopimelic acid, 25 mg/L of tetracycline, 25 mg/L of chloramphenicol, 30 g/L of L-glutamic acid, adjusted to pH 4.5 with aqueous ammonia) plate. A colony emerged after culture at 32°C for 2 days was obtained as a strain with high-concentration Glu-resistance at low pH.

For the obtained strain, growth level in M9-E liquid medium was measured and L-glutamic acid-producing ability was tested in a 50-ml volume large test tube containing 5 ml of L-glutamic acid production test medium (40 g/L of glucose, 20 g/L of ammonium sulfate, 0.5 g/L of magnesium sulfate heptahydrate, 2 g/L of potassium dihydrogenphosphate, 0.5 g/L of sodium chloride, 0.25 g/L of calcium chloride dihydrate, 0.02 g/L of ferrous sulfate heptahydrate, 0.02 g/L of manganese sulfate tetrahydrate, 0.72 mg/L of zinc sulfate dihydrate, 0.64 mg/L of copper sulfate pentahydrate, 0.72 mg/L of cobalt chloride hexahydrate, 0.4 mg/L of boric acid, 1.2 mg/L of sodium molybdate dihydrate, 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol). A strain that exhibited the best growth level and the same L-glutamic acid-producing ability as that of its parent strain, the SC17/RSFCPG+pSTVCB strain, was designated as *Enterobacter agglomerans* AJ13601. The

AJ13601 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (now, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology; Central 6, Higashi 1-1-1, Tsukuba-shi, Ibaraki 305-8566, Japan) on August 18, 1999 and received an accession number of FERM P-17516. It was then transferred to an international deposition under the provisions of Budapest Treaty on July 6, 2000 and received an accession number of FERM BP-7207.

Example 1

The *Enterobacter agglomerans* AJ13601 strain was cultured on LBG agar medium (10 g/L of trypton, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose and 15 g/L of agar) containing 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol at 30°C for 14 hours, and the cells in one plate (diameter: 8.5 cm) were collected and inoculated into 300 mL of a culture medium containing 50 g/L of glucose, 4 g/L of ammonium sulfate, 0.4 g/L of magnesium sulfate heptahydrate, 2 g/L of monopotassium dihydrogenphosphate, 10 mg/L of ferrous sulfate heptahydrate, 10 mg/L of manganese sulfate pentahydrate, 4 g/L of yeast extract, 400 mg/L of L-lysine hydrochloride, 400 mg/L of DL-methionine, 400 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline

hydrochloride and 25 mg/L of chloramphenicol, in a 1 L-volume jar fermenter to start culture at 34°C and at pH 6.0. Culture pH was controlled by adding ammonia gas. The culture was terminated about 16 hours after beginning of the culture, at a phase in which the glucose in the culture medium was depleted.

15 mL of the culture broth cultured as described above was inoculated into 15 L of a culture medium containing 50 g/L of glucose, 4 g/L of ammonium sulfate, 0.4 g/L of magnesium sulfate heptahydrate, 2 g/L of monopotassium dihydrogenphosphate, 10 mg/L of ferrous sulfate heptahydrate, 10 mg/L of manganese sulfate pentahydrate, 4 g/L of yeast extract, 400 mg/L of L-lysine hydrochloride, 400 mg/L of DL-methionine, 400 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, in a 30 L-volume jar fermenter to start culture at 34°C and at pH 6.0. Culture pH was controlled by adding ammonia gas. The culture was terminated about 16 hours after beginning of the culture, at a phase in which the glucose in the culture medium was depleted.

2.8 L of the culture broth cultured as described above was inoculated into 14 L of a culture medium containing 50 g/L of glucose, 5 g/L of ammonium sulfate, 0.4 g/L of magnesium sulfate heptahydrate, 5 g/L of monopotassium dihydrogenphosphate, 20 mg/L of ferrous sulfate heptahydrate, 20 mg/L of manganese sulfate pentahydrate, 6 g/L of yeast extract, 800 mg/L of L-

lysine hydrochloride, 600 mg/L of DL-methionine, 600 mg/L of DL-α,ε-diaminopimelic acid, 1.5 g/L of sodium chloride, 0.75 g/L of calcium chloride dihydrate, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, in a 30 L-volume jar fermenter to start culture at 34°C and at pH 6.0. While the culture was continued, as L-glutamic acid accumulated, pH spontaneously decreased to reach pH 4.5. Thereafter, the culture pH was controlled to be pH 4.5 by adding ammonia gas. After the initially-added glucose was depleted, 700 g/L of an aqueous solution of glucose was continuously added.

The fermentative production of L-glutamic acid was continued as described above. When the concentration of L-glutamic acid accumulated in the culture broth reached 45 g/L, 30 g of L-glutamic acid crystals of α-form were added to the culture broth as a suspension of crystals in 100 ml water from the upper part of the jar fermenter, and the culture was further continued. The culture was terminated at a phase in which a sum of a concentration of L-glutamic acid accumulated as crystals in the medium and a concentration of L-glutamic acid dissolved in the medium reached 100 g/L. A considerable amount of L-glutamic acid crystals of α-form precipitated in the jar fermenter. By using a method of adding sulfuric acid to the medium, pH was adjusted to 3.2 at which solubility of L-glutamic acid becomes low. Further, crystallization of L-glutamic acid dissolved in the

solution was facilitated by cooling, to obtain crystal slurry. Crystals of L-glutamic acid which precipitated in the crystal slurry were separated by a superdecanter, to obtain a desired organic nitrogen-containing composition.

Analytical content of each ingredient with respect to total solid matter in the resultant organic nitrogen-containing composition is shown in Table 1.

Table 1

Analytical values of organic nitrogen-containing composition

Ingredient	wt% with respect to total solid matter
Total nitrogen	13.1%
(of the above) Ammonia nitrogen	7.1%
(of the above) Organic nitrogen	6.0%
(of the above) Glutamic acid nitrogen	2.2%
(of the above) Other nitrogen	3.8%
(Mass% of organic nitrogen with respect to total nitrogen	46%)
Sulfate group	18.9%
(Mass% of sulfate group with respect to organic nitrogen	315%)

Comparative Example 1

The culture was performed in the same way as in Example 1 except that the culture condition in the 30-L

jar fermenter which contained 300 mL medium in Example 1 was changed as follows: the culture was started at 34°C and at pH 6.0 and then the culture pH was controlled to maintain pH 6.0 by adding ammonia gas. From the resultant medium, an organic nitrogen-containing composition was obtained by the same way as in Example 1.

Content of each ingredient with respect to total solid matter in the resultant organic nitrogen-containing composition is shown in Table 2.

Table 2

Analytical values of control organic nitrogen-containing composition

Ingredient	wt% with respect to total solid matter
Total nitrogen	16.2%
(of the above) Ammonia nitrogen	12.2%
(of the above) Organic nitrogen	4.0%
(of the above) Glutamic acid nitrogen	1.4%
(of the above) Other nitrogen	2.6%
(Mass% of organic nitrogen with respect to total nitrogen	25%)
Sulfate group	38.2%
(Mass% of sulfate group with respect to organic nitrogen	955%)

From the results in Table 1 and Table 2, it cam be seen that the organic nitrogen-containing composition of the present invention has a high content of organic nitrogen which is effective as a fertilizer, a high proportion of organic nitrogen with respect to total nitrogen, and, in particular, a high content of organic nitrogen other than glutamic acid nitrogen. Also, it can be seen that the amount of sulfate group is small and, therefore, the organic nitrogen-containing composition of the present invention is suitable for a raw material of a fertilizer.

SEQUENCE LISTING

<110> Takayuki Koda
Kazuhiro Sato

<120> ORGANIC NITROGEN-CONTAINING COMPOSITION AND FERTILIZER COMPRISING THE SAME

<130>

<140> JP 2001-44137
<141> 2001-02-20

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<211> 935
<212> PRT
<213> Enterobacter agglomerans

<400> 1

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Met Gln Asn Ser Ala Met Lys Pro Trp Leu Asp Ser Ser Trp Leu Ala
  1               5                  10                  15
Gly Ala Asn Gln Ser Tyr Ile Glu Gln Leu Tyr Glu Asp Phe Leu Thr
             20                  25                  30
Asp Pro Asp Ser Val Asp Ala Val Trp Arg Ser Met Phe Gln Gln Leu
         35                  40                  45
Pro Gly Thr Gly Val Lys Pro Glu Gln Phe His Ser Ala Thr Arg Glu
     50                  55                  60
Tyr Phe Arg Arg Leu Ala Lys Asp Ala Ser Arg Tyr Thr Ser Ser Val
 65                  70                  75                  80
Thr Asp Pro Ala Thr Asn Ser Lys Gln Val Lys Val Leu Gln Leu Ile
                 85                  90                  95
Asn Ala Phe Arg Phe Arg Gly His Gln Glu Ala Asn Leu Asp Pro Leu
            100                 105                 110
Gly Leu Trp Lys Gln Asp Arg Val Ala Asp Leu Asp Pro Ala Phe His
        115                 120                 125
Asp Leu Thr Asp Ala Asp Phe Gln Glu Ser Phe Asn Val Gly Ser Phe
    130                 135                 140
Ala Ile Gly Lys Glu Thr Met Lys Leu Ala Asp Leu Phe Asp Ala Leu
145                 150                 155                 160
Lys Gln Thr Tyr Cys Gly Ser Ile Gly Ala Glu Tyr Met His Ile Asn
                165                 170                 175
Asn Thr Glu Glu Lys Arg Trp Ile Gln Gln Arg Ile Glu Ser Gly Ala
            180                 185                 190
Ser Gln Thr Ser Phe Ser Gly Glu Glu Lys Lys Gly Phe Leu Lys Glu
        195                 200                 205
Leu Thr Ala Ala Glu Gly Leu Glu Lys Tyr Leu Gly Ala Lys Phe Pro
    210                 215                 220
Gly Ala Lys Arg Phe Ser Leu Glu Gly Gly Asp Ala Leu Val Pro Met
225                 230                 235                 240
Leu Arg Glu Met Ile Arg His Ala Gly Lys Ser Gly Thr Arg Glu Val
                245                 250                 255
Val Leu Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Ile Asn Val
            260                 265                 270
```

Leu Gly Lys Lys Pro Gln Asp Leu Phe Asp Glu Phe Ser Gly Lys His
275 280 285
Lys Glu His Leu Gly Thr Gly Asp Val Lys Tyr His Met Gly Phe Ser
290 295 300
Ser Asp Ile Glu Thr Glu Gly Gly Leu Val His Leu Ala Leu Ala Phe
305 310 315 320
Asn Pro Ser His Leu Glu Ile Val Ser Pro Val Val Met Gly Ser Val
325 330 335
Arg Ala Arg Leu Asp Arg Leu Ala Glu Pro Val Ser Asn Lys Val Leu
340 345 350
Pro Ile Thr Ile His Gly Asp Ala Ala Val Ile Gly Gln Gly Val Val
355 360 365
Gln Glu Thr Leu Asn Met Ser Gln Ala Arg Gly Tyr Glu Val Gly Gly
370 375 380
Thr Val Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn
385 390 395 400
Pro Lys Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met
405 410 415
Val Leu Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val
420 425 430
Ala Phe Val Thr Arg Leu Ala Leu Asp Tyr Arg Asn Thr Phe Lys Arg
435 440 445
Asp Val Phe Ile Asp Leu Val Cys Tyr Arg Arg His Gly His Asn Glu
450 455 460
Ala Asp Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys
465 470 475 480
Lys His Pro Thr Pro Arg Lys Ile Tyr Ala Asp Arg Leu Glu Gly Glu
485 490 495
Gly Val Ala Ser Gln Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg
500 505 510
Asp Ala Leu Asp Ala Gly Glu Cys Val Val Pro Glu Trp Arg Pro Met
515 520 525
Ser Leu His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp
530 535 540
Glu Pro Tyr Pro Ala Gln Val Asp Met Lys Arg Leu Lys Glu Leu Ala
545 550 555 560
Leu Arg Ile Ser Gln Val Pro Glu Gln Ile Glu Val Gln Ser Arg Val
565 570 575
Ala Lys Ile Tyr Asn Asp Arg Lys Leu Met Ala Glu Gly Glu Lys Ala
580 585 590
Phe Asp Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp
595 600 605
Glu Gly Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr
610 615 620
Phe Phe His Arg His Ala Val Val His Asn Gln Ala Asn Gly Ser Thr
625 630 635 640
Tyr Thr Pro Leu His His Ile His Asn Ser Gln Gly Glu Phe Lys Val
645 650 655
Trp Asp Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly
660 665 670
Tyr Ala Thr Ala Glu Pro Arg Val Leu Thr Ile Trp Glu Ala Gln Phe
675 680 685
Gly Asp Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser
690 695 700
Ser Gly Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu

```
705                 710                 715                 720
Pro His Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu
                725                 730                 735
Glu Arg Tyr Leu Gln Leu Cys Ala Glu Gln Asn Met Gln Val Cys Val
            740                 745                 750
Pro Ser Thr Pro Ala Gln Val Tyr His Met Leu Arg Arg Gln Ala Leu
        755                 760                 765
Arg Gly Met Arg Arg Pro Leu Val Val Met Ser Pro Lys Ser Leu Leu
    770                 775                 780
Arg His Pro Leu Ala Ile Ser Ser Leu Asp Glu Leu Ala Asn Gly Ser
785                 790                 795                 800
Phe Gln Pro Ala Ile Gly Glu Ile Asp Asp Leu Asp Pro Gln Gly Val
                805                 810                 815
Lys Arg Val Val Leu Cys Ser Gly Lys Val Tyr Tyr Asp Leu Leu Glu
            820                 825                 830
Gln Arg Arg Lys Asp Glu Lys Thr Asp Val Ala Ile Val Arg Ile Glu
        835                 840                 845
Gln Leu Tyr Pro Phe Pro His Gln Ala Val Gln Glu Ala Leu Lys Ala
    850                 855                 860
Tyr Ser His Val Gln Asp Phe Val Trp Cys Gln Glu Glu Pro Leu Asn
865                 870                 875                 880
Gln Gly Ala Trp Tyr Cys Ser Gln His His Phe Arg Asp Val Val Pro
                885                 890                 895
Phe Gly Ala Thr Leu Arg Tyr Ala Gly Arg Pro Ala Ser Ala Ser Pro
            900                 905                 910
Ala Val Gly Tyr Met Ser Val His Gln Gln Gln Gln Gln Asp Leu Val
        915                 920                 925
Asn Asp Ala Leu Asn Val Asn
    930                 935
```

<210> 2
<211> 407
<212> PRT
<213> Enterobacter agglomerans

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<400> 2
Met Ser Ser Val Asp Ile Leu Val Pro Asp Leu Pro Glu Ser Val Ala
  1               5                  10                  15
Asp Ala Thr Val Ala Thr Trp His Lys Lys Pro Gly Asp Ala Val Ser
             20                  25                  30
Arg Asp Glu Val Ile Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu
         35                  40                  45
Val Pro Ala Ser Ala Asp Gly Val Leu Glu Ala Val Leu Glu Asp Glu
     50                  55                  60
Gly Ala Thr Val Thr Ser Arg Gln Ile Leu Gly Arg Leu Lys Glu Gly
 65                  70                  75                  80
Asn Ser Ala Gly Lys Glu Ser Ser Ala Lys Ala Glu Ser Asn Asp Thr
                 85                  90                  95
Thr Pro Ala Gln Arg Gln Thr Ala Ser Leu Glu Glu Glu Ser Ser Asp
            100                 105                 110
Ala Leu Ser Pro Ala Ile Arg Arg Leu Ile Ala Glu His Asn Leu Asp
        115                 120                 125
Ala Ala Gln Ile Lys Gly Thr Gly Val Gly Gly Arg Leu Thr Arg Glu
    130                 135                 140
Asp Val Glu Lys His Leu Ala Asn Lys Pro Gln Ala Glu Lys Ala Ala
```

```
145                 150                 155                 160
Ala Pro Ala Ala Gly Ala Ala Thr Ala Gln Gln Pro Val Ala Asn Arg
                165                 170                 175
Ser Glu Lys Arg Val Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu
            180                 185                 190
Arg Leu Leu Glu Ala Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn
        195                 200                 205
Glu Ile Asn Met Lys Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Asp
    210                 215                 220
Ala Phe Glu Lys Arg His Gly Val Arg Leu Gly Phe Met Ser Phe Tyr
225                 230                 235                 240
Ile Lys Ala Val Val Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala
                245                 250                 255
Ser Ile Asp Gly Glu Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser
            260                 265                 270
Ile Ala Val Ser Thr Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp
        275                 280                 285
Val Asp Ala Leu Ser Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu
    290                 295                 300
Ala Val Lys Gly Arg Asp Gly Lys Leu Thr Val Asp Asp Leu Thr Gly
305                 310                 315                 320
Gly Asn Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser
                325                 330                 335
Thr Pro Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala
            340                 345                 350
Ile Lys Asp Arg Pro Met Ala Val Asn Gly Gln Val Val Ile Leu Pro
        355                 360                 365
Met Met Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg
    370                 375                 380
Glu Ser Val Gly Tyr Leu Val Ala Val Lys Glu Met Leu Glu Asp Pro
385                 390                 395                 400
Ala Arg Leu Leu Leu Asp Val
                405
```

<210> 3
<211> 41
<212> PRT
<213> Enterobacter agglomerans

<400> 3

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Met Asn Leu His Glu Tyr Gln Ala Lys Gln Leu Phe Ala Arg Tyr Gly
  1               5                  10                  15
Met Pro Ala Pro Thr Gly Tyr Ala Cys Thr Thr Pro Arg Glu Ala Glu
            20                  25                  30
Glu Ala Ala Ser Lys Ile Gly Ala Gly
        35                  40
```

<210> 4
<211> 39
<212> PRT
<213> Enterobacter agglomerans

<400> 4

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Ala Phe Ser Val Phe Arg Cys His Ser Ile Met Asn Cys Val Ser Val
  1               5                  10                  15
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Cys Pro Lys Gly Leu Asn Pro Thr Arg Ala Ile Gly His Ile Lys Ser
20 25 30
Met Leu Leu Gln Arg Ser Ala
35

<210> 5
<211> 933
<212> PRT
<213> Escherichia coli

<400> 5
Met Gln Asn Ser Ala Leu Lys Ala Trp Leu Asp Ser Ser Tyr Leu Ser
1 5 10 15
Gly Ala Asn Gln Ser Trp Ile Glu Gln Leu Tyr Glu Asp Phe Leu Thr
20 25 30
Asp Pro Asp Ser Val Asp Ala Asn Trp Arg Ser Thr Phe Gln Gln Leu
35 40 45
Pro Gly Thr Gly Val Lys Pro Asp Gln Phe His Ser Gln Thr Arg Glu
50 55 60
Tyr Phe Arg Arg Leu Ala Lys Asp Ala Ser Arg Tyr Ser Ser Thr Ile
65 70 75 80
Ser Asp Pro Asp Thr Asn Val Lys Gln Val Lys Val Leu Gln Leu Ile
85 90 95
Asn Ala Tyr Arg Phe Arg Gly His Gln His Ala Asn Leu Asp Pro Leu
100 105 110
Gly Leu Trp Gln Gln Asp Lys Val Ala Asp Leu Asp Pro Ser Phe His
115 120 125
Asp Leu Thr Glu Ala Asp Phe Gln Glu Thr Phe Asn Val Gly Ser Phe
130 135 140
Ala Ser Gly Lys Glu Thr Met Lys Leu Gly Glu Leu Leu Glu Ala Leu
145 150 155 160
Lys Gln Thr Tyr Cys Gly Pro Ile Gly Ala Glu Tyr Met His Ile Thr
165 170 175
Ser Thr Glu Glu Lys Arg Trp Ile Gln Gln Arg Ile Glu Ser Gly Arg
180 185 190
Ala Thr Phe Asn Ser Glu Glu Lys Lys Arg Phe Leu Ser Glu Leu Thr
195 200 205
Ala Ala Glu Gly Leu Glu Arg Tyr Leu Gly Ala Lys Phe Pro Gly Ala
210 215 220
Lys Arg Phe Ser Leu Glu Gly Gly Asp Ala Leu Ile Pro Met Leu Lys
225 230 235 240
Glu Met Ile Arg His Ala Gly Asn Ser Gly Thr Arg Glu Val Val Leu
245 250 255
Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Val Asn Val Leu Gly
260 265 270
Lys Lys Pro Gln Asp Leu Phe Asp Glu Phe Ala Gly Lys His Lys Glu
275 280 285
His Leu Gly Thr Gly Asp Val Lys Tyr His Met Gly Phe Ser Ser Asp
290 295 300
Phe Gln Thr Asp Gly Gly Leu Val His Leu Ala Leu Ala Phe Asn Pro
305 310 315 320
Ser His Leu Glu Ile Val Ser Pro Val Val Ile Gly Ser Val Arg Ala
325 330 335
Arg Leu Asp Arg Leu Asp Glu Pro Ser Ser Asn Lys Val Leu Pro Ile
340 345 350

Thr Ile His Gly Asp Ala Ala Val Thr Gly Gln Gly Val Val Gln Glu
355 360 365
Thr Leu Asn Met Ser Lys Ala Arg Gly Tyr Glu Val Gly Gly Thr Val
370 375 380
Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn Pro Leu
385 390 395 400
Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met Val Gln
405 410 415
Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val Ala Phe
420 425 430
Val Thr Arg Leu Ala Leu Asp Phe Arg Asn Thr Phe Lys Arg Asp Val
435 440 445
Phe Ile Asp Leu Val Ser Tyr Arg Arg His Gly His Asn Glu Ala Asp
450 455 460
Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys Lys His
465 470 475 480
Pro Thr Pro Arg Lys Ile Tyr Ala Asp Lys Leu Glu Gln Glu Lys Val
485 490 495
Ala Thr Leu Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg Asp Ala
500 505 510
Leu Asp Ala Gly Asp Cys Val Val Ala Glu Trp Arg Pro Met Asn Met
515 520 525
His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp Glu Glu
530 535 540
Tyr Pro Asn Lys Val Glu Met Lys Arg Leu Gln Glu Leu Ala Lys Arg
545 550 555 560
Ile Ser Thr Val Pro Glu Ala Val Glu Met Gln Ser Arg Val Ala Lys
565 570 575
Ile Tyr Gly Asp Arg Gln Ala Met Ala Ala Gly Glu Lys Leu Phe Asp
580 585 590
Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp Glu Gly
595 600 605
Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr Phe Phe
610 615 620
His Arg His Ala Val Ile His Asn Gln Ser Asn Gly Ser Thr Tyr Thr
625 630 635 640
Pro Leu Gln His Ile His Asn Gly Gln Gly Ala Phe Arg Val Trp Asp
645 650 655
Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly Tyr Ala
660 665 670
Thr Ala Glu Pro Arg Thr Leu Thr Ile Trp Glu Ala Gln Phe Gly Asp
675 680 685
Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser Ser Gly
690 695 700
Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu Pro His
705 710 715 720
Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu Glu Arg
725 730 735
Tyr Leu Gln Leu Cys Ala Glu Gln Asn Met Gln Val Cys Val Pro Ser
740 745 750
Thr Pro Ala Gln Val Tyr His Met Leu Arg Arg Gln Ala Leu Arg Gly
755 760 765
Met Arg Arg Pro Leu Val Val Met Ser Pro Lys Ser Leu Leu Arg His
770 775 780
Pro Leu Ala Val Ser Ser Leu Glu Glu Leu Ala Asn Gly Thr Phe Leu

785 790 795 800
Pro Ala Ile Gly Glu Ile Asp Glu Leu Asp Pro Lys Gly Val Lys Arg
805 810 815
Val Val Met Cys Ser Gly Lys Val Tyr Tyr Asp Leu Leu Glu Gln Arg
820 825 830
Arg Lys Asn Asn Gln His Asp Val Ala Ile Val Arg Ile Glu Gln Leu
835 840 845
Tyr Pro Phe Pro His Lys Ala Met Gln Glu Val Leu Gln Gln Phe Ala
850 855 860
His Val Lys Asp Phe Val Trp Cys Gln Glu Glu Pro Leu Asn Gln Gly
865 870 875 880
Ala Trp Tyr Cys Ser Gln His His Phe Arg Glu Val Ile Pro Phe Gly
885 890 895
Ala Ser Leu Arg Tyr Ala Gly Arg Pro Ala Ser Ala Ser Pro Ala Val
900 905 910
Gly Tyr Met Ser Val His Gln Lys Gln Gln Gln Asp Leu Val Asn Asp
915 920 925
Ala Leu Asn Val Glu
930

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<211> 405
<212> PRT
<213> Escherichia coli

<400> 6
Met Ser Ser Val Asp Ile Leu Val Pro Asp Leu Pro Glu Ser Val Ala
1 5 10 15
Asp Ala Thr Val Ala Thr Trp His Lys Lys Pro Gly Asp Ala Val Val
20 25 30
Arg Asp Glu Val Leu Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu
35 40 45
Val Pro Ala Ser Ala Asp Gly Ile Leu Asp Ala Val Leu Glu Asp Glu
50 55 60
Gly Thr Thr Val Thr Ser Arg Gln Ile Leu Gly Arg Leu Arg Glu Gly
65 70 75 80
Asn Ser Ala Gly Lys Glu Thr Ser Ala Lys Ser Glu Glu Lys Ala Ser
85 90 95
Thr Pro Ala Gln Arg Gln Gln Ala Ser Leu Glu Glu Gln Asn Asn Asp
100 105 110
Ala Leu Ser Pro Ala Ile Arg Arg Leu Leu Ala Glu His Asn Leu Asp
115 120 125
Ala Ser Ala Ile Lys Gly Thr Gly Val Gly Gly Arg Leu Thr Arg Glu
130 135 140
Asp Val Glu Lys His Leu Ala Lys Ala Pro Ala Lys Glu Ser Ala Pro
145 150 155 160
Ala Ala Ala Ala Pro Ala Ala Gln Pro Ala Leu Ala Ala Arg Ser Glu
165 170 175
Lys Arg Val Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu Arg Leu
180 185 190
Leu Glu Ala Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn Glu Val
195 200 205
Asn Met Lys Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Glu Ala Phe
210 215 220
Glu Lys Arg His Gly Ile Arg Leu Gly Phe Met Ser Phe Tyr Val Lys

225 230 235 240
Ala Val Val Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala Ser Ile
245 250 255
Asp Gly Asp Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser Met Ala
260 265 270
Val Ser Thr Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp Val Asp
275 280 285
Thr Leu Gly Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu Ala Val
290 295 300
Lys Gly Arg Asp Gly Lys Leu Thr Val Glu Asp Leu Thr Gly Gly Asn
305 310 315 320
Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser Thr Pro
325 330 335
Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala Ile Lys
340 345 350
Asp Arg Pro Met Ala Val Asn Gly Gln Val Glu Ile Leu Pro Met Met
355 360 365
Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg Glu Ser
370 375 380
Val Gly Phe Leu Val Thr Ile Lys Glu Leu Leu Glu Asp Pro Thr Arg
385 390 395 400
Leu Leu Leu Asp Val
405

<210> 7
<211> 60
<212> PRT
<213> Escherichia coli

<400> 7
Met Asn Leu His Glu Tyr Gln Ala Lys Gln Leu Phe Ala Arg Tyr Gly
1 5 10 15
Leu Pro Ala Pro Val Gly Tyr Ala Cys Thr Thr Pro Arg Glu Ala Glu
20 25 30
Glu Ala Ala Ser Lys Ile Gly Ala Gly Pro Trp Val Val Lys Cys Gln
35 40 45
Val His Ala Gly Gly Arg Gly Lys Ala Gly Gly Val
50 55 60

<210> 8
<211> 58
<212> PRT
<213> Escherichia coli

<400> 8
Phe Leu Ile Asp Ser Arg Asp Thr Glu Thr Asp Ser Arg Leu Asp Gly
1 5 10 15
Leu Ser Asp Ala Phe Ser Val Phe Arg Cys His Ser Ile Met Asn Cys
20 25 30
Val Ser Val Cys Pro Lys Gly Leu Asn Pro Thr Arg Ala Ile Gly His
35 40 45
Ile Lys Ser Met Leu Leu Gln Arg Asn Ala
50 55